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In Vitro and In Vivo Photosensitizing Capabilities of 5-ALA Versus Photofrin® in Vascular Endothelial Cells

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Background and Objective: **The objective of the present study was to evaluate the feasibility of photodynamic therapy (PDT) for complicated hemangiomas. The photosensitizing activities of 5-aminolevulinic acid (5-ALA) and Photofrin® were evaluated in vitro with human dermal microvascular endothelial cells (MEC) and in vivo with the chicken cox comb.**
Study Design/Materials and Methods: **The in vitro absorption and photosensitizing activities of 5-ALA and Photofrin® were examined in a MEC culture system. The percentages of MEC killed by different drug concentrations at a wavelength of 630 nm were measured by either live/dead or lactate dehydrogenase-released assays. Similarly, the in vivo biological activities of 5-ALA and Photofrin® exposed to different total light dosages at 630 nm were studied by determining the amount of necrosis produced in chicken combs.**
Results: **MEC incubated with 5-ALA at a concentration of 35 μg/ml and exposed to laser light at 630 nm at a power density of 100 mW/cm² showed a 50% cell kill. MEC incubated with Photofrin® at a concentration of 3.5 μg/ml and exposed to laser light at 630 nm at a power density of 100 mW/cm² showed a 50% cell kill. Chicken combs that received 200 mg/kg of 5-ALA exposed to laser light at 630 nm at a power density of 100 mW/cm² had an injury depth of 362.5 ± 27.6 μm at histologic examination. Combs exposed to a power density of 100 or 120 mW/cm² showed injury depths of 732.5 ± 29.1 and 792.5 ± 36.0 μm, respectively. Chicken combs that received 2.5 mg/kg of Photofrin® exposed to laser light at 630 nm at a power density of 80 mW/cm² had an injury depth of 535.6 ± 22.3 μm at histologic examination. Combs exposed to a power density of 100 or 120 mW/cm² showed injury depths of 795.8 ± 32.5 and 805.2 ± 49.1 μm, respectively.**
Conclusion: **Both 5-ALA and Photofrin® have the capability to destroy MEC in vitro and vasculature in vivo. However, Photo-**

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frin® achieved a higher degree of cell kill and tissue destruction at lower drug concentrations and at lower power densities. Lasers Surg. Med. 24:178–186, 1999. © 1999 Wiley-Liss, Inc.

Key words: microvascular endothelial cells (MEC); PDT; 5-ALA; Photofrin®

INTRODUCTION

Hemangioma at infancy (strawberry hemangioma) is a common birthmark that occurs on many anatomical sites [1]. The condition is self-limiting in that the majority of hemangiomas will spontaneously involute by age 3–7 years [2,3]. When obstruction of body orifices, ulceration, or bleeding occurs, these otherwise benign lesions can cause considerable morbidity.

Different alternative methods of treatment such as carbon dioxide snow, liquid nitrogen, radiation therapy, steroid therapy, or interferon 2-a have been used with inevitable complications [4–8]. Lasers have also become one of the major tools to treat hemangiomas. The CO_2 laser has been used for excision or vaporization of small hemangiomas. The argon laser delivers a combination of wavelengths (488 and 514 nm) that are absorbed by oxyhemoglobin present in the vasculature. The argon laser has been used for treatment of many vascular lesions, including port-wine stains, telangiectasias, and venous lakes [9,10]. Its use has been controversial, with the risk of scarring noted as the primary drawback [11–14]. However, argon laser treatment of hemangiomas has been recommended for complicated lesions or small hemangiomas [12]. The potassium-titanyl-phosphate (KTP) laser at a wavelength of 532 nm and the neodymium:yttrium-aluminum-garnet (Nd:YAG) laser at a wavelength of 1,064 nm are alternative choices under specific circumstances. For small hemangiomas, the lesions are excised completely by the KTP laser (532 nm), with primary wound closure. The Nd:YAG laser can produce effects at tissue depths of 4–6 mm into the dermis, resulting in a large volume of coagulated tissue. However, profound thermal damage can be produced unknowingly by an inexperienced physician. The Nd:YAG laser has also been used for intralesional photocoagulation [15]. The flashlamp-pumped pulsed dye laser at a wavelength of 577 or 585 nm or the argon tunable dye laser (Coherent Medical, Palo Alto, CA) with a Hexascan (Lihtan Technologies, San Rafael, CA) attachment could be considered for treating hemangiomas prior to full involution. These are useful therapeutic approaches for superficial hemangiomas or those hemangiomas that are slow to regress in older children [16].

The basic concept of photodynamic therapy (PDT) is that certain molecules can function as photosensitizers [17]. The presence of these photosensitizers in biological tissue makes the tissue vulnerable to light at wavelengths absorbed by the chromophore. Previous basic science studies have attempted to achieve an understanding of the mechanism of tumor destruction after PDT [18,19]. Histopathology of PDT-treated tumors shows that apparent internal hemorrhage and red cell extravasation are common findings after PDT. The rationale for our use of PDT in hypervascular cutaneous anomalies is based on the fact that PDT will allow the destruction of the targeted blood vessels buried deep within the skin without the production of heat. Therefore, risks inherent in conventional photothermal laser therapy, such as hypertrophic scarring, changes in skin pigmentation, atrophy, or induration, should not occur.

MATERIALS AND METHODS

Photosensitizers

5-Aminolevulinic acid (5-ALA), obtained from Sigma Chemical Co. (St. Louis, MO), and Photofrin®, obtained from Quadra Logic Technologies, Inc. (Vancouver, BC, Canada), were stored in the dark at 4°C. 5-ALA as hydrochloride in 98% pure powder was prepared by dissolution in Dullbeco's phosphate buffered saline (PBS) to a final concentration of 100 μg/ml at pH 5.6. Photofrin® was prepared with 5% dextrose to a final concentration of 2.5 μg/ml. Subcellular localizations were determined 3 h after incubation by using computer-enhanced low-light level digital video fluorescence microscopy. The 630-nm peak was selected for laser irradiation for both photosensitizers because of the improved tissue penetration of light at this longer wavelength [20].

Microvascular Endothelial Cell (MEC) Culture System

Well-preserved fresh human foreskins were harvested, and the connective tissue and vessels were separated by dissection under a microscope.

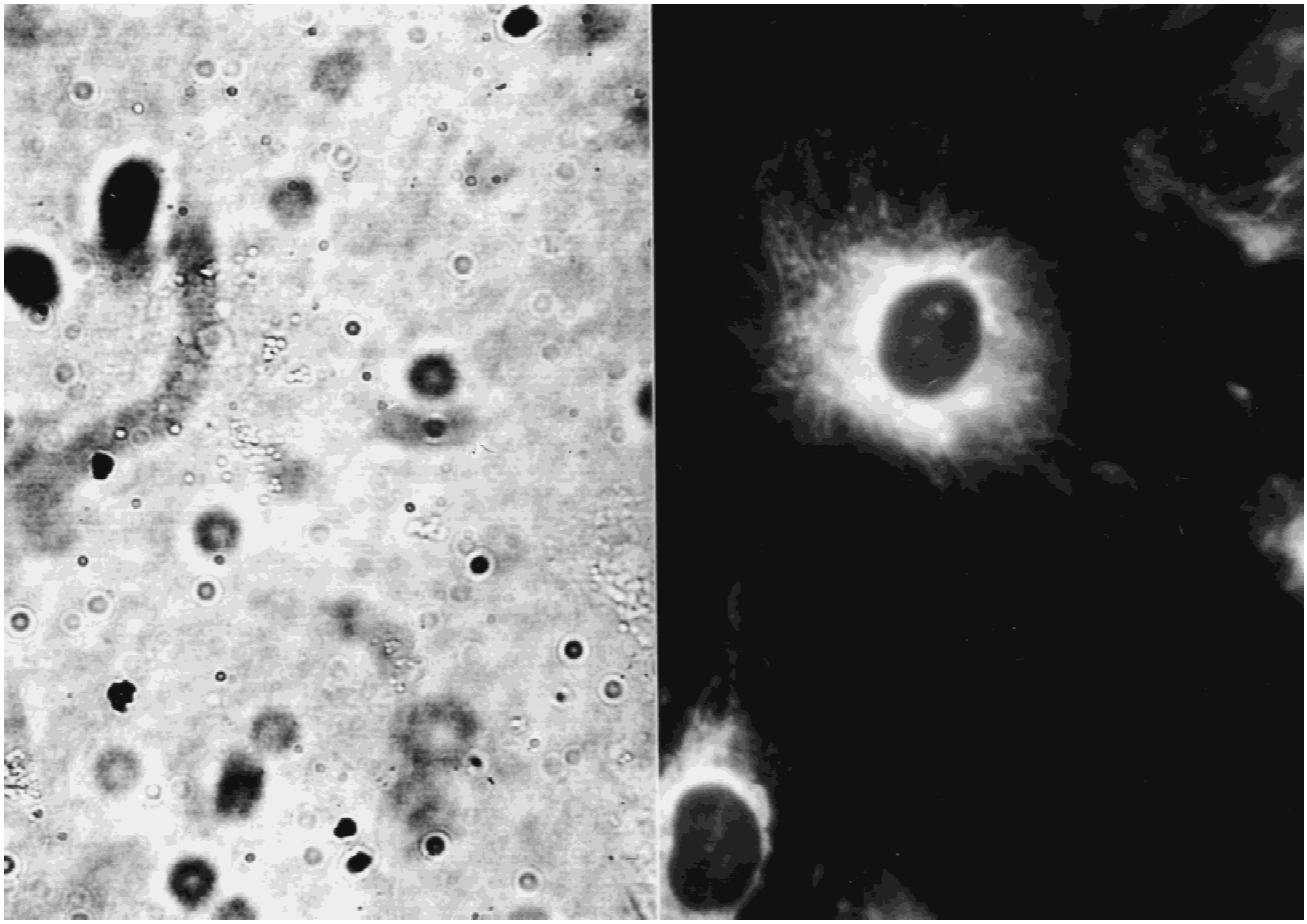


Fig. 1. Under a fluorescent microscope, subcellular localization of 5-aminolevulinic acid in mitochondria is shown when the concentration is greater than 35 μg/ml.

The vascular structures are readily accessible, and the endothelial cells may be directly released by brief incubation with dispase in 7.5% CO_2 for 3 h at 37°C. To remove excess dispase, the tissue was washed with PBS. The vascular tissue was transferred to fresh medium by the application of moderate pressure on the vessel wall with a blunt scalpel blade. The liberated MEC were pelted by centrifugation (1,000 rpm) for 5 min, resuspended in dermal MEC proliferation medium (Cell Applications, Inc., San Diego, CA) and plated on fibronectin-coated T25 flasks. The von Willebrand factor and hematopoietic progenitor antigen CD36 were detected to characterize MEC.

Fresh human proliferative hemangiomas were harvested by surgical excision and prepared for MEC by using the same procedure as used for foreskins. The characteristics (von Willebrand factor, CD36) of MEC from both the foreskins and hemangiomas were confirmed to be identical. One week before photosensitizer incubation, the MEC were subcultured by using 1 ml 0.3% trypsin–1% EDTA (Cell Applications, Inc.) in each T25 flask in 7.5% CO_2 for 5 min at 37°C. The MEC were then neutralized with the same amount of trypsin neutralizing solution (Cell Applications, Inc.) and plated on a 24-well plate coated with attachment factor solution (Cell Applications, Inc.) at a density of 2.5–3.5 × 10^4. On the day of photosensitizer incubation, MEC were incubated with 5-ALA at a concentration of 0, 5, 10, 25, 50, or 100 μg/ml for 3 h. The MEC were washed with fresh PBS and exposed to the laser (λ = 630 nm). These cells received 10 J/cm^2 at a power density of 100 mW/cm^2. Similarly, MEC were also prepared in a 24-well plate for treatment with Photofrin® at a concentration of 0, 1, 2.5, or 5 μg/ml and irradiated with a wavelength of 630 nm at an energy density of 10 J/cm^2 and a power density of 100 mW/cm^2, as described above.

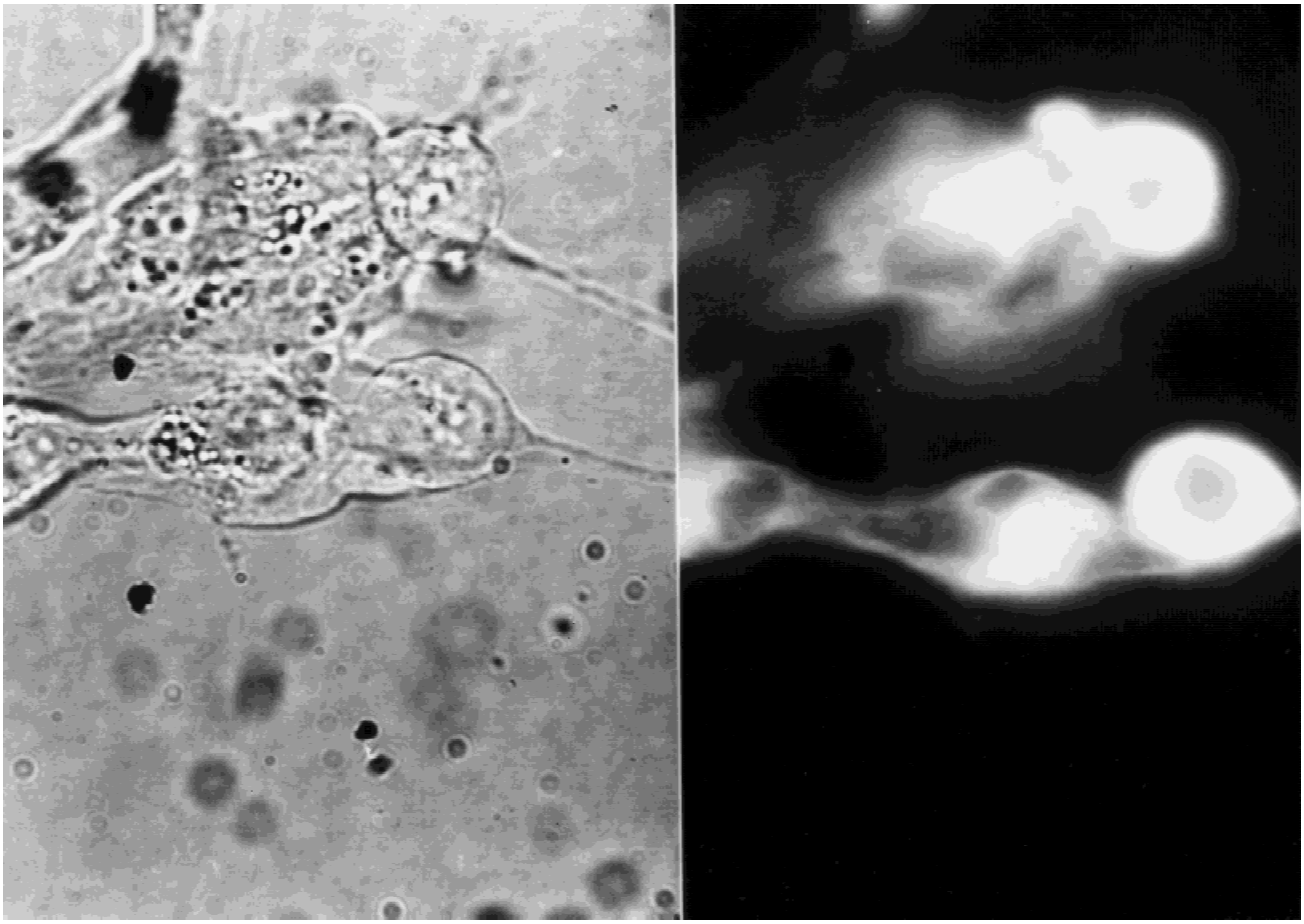


Fig. 2. Under a fluorescent microscope, subcellular localization of Photofrin® throughout cytoplasm is shown when the concentration is greater than 3.5 μg/ml.

Live/Dead Assay

Twenty-four hours after laser irradiation, the cytoplasmic microtubule (MT) status of the MEC was monitored by immunofluorescence microscopy by using tubulin antibody and rhodamine-phalloidin (Molecular Probes, Eugene, OR); a viability cytotoxicity kit (Molecular Probes, Inc.), a two-color fluorescence assay, was used to determine the percentage cell kill by counting the number of dead cells per 200 cells within the 1.5-cm-diameter irradiation ring under the fluorescence microscope. The differential permeability of live and dead cells to a pair of fluorescence stains allows live and dead cells to appear as fluorescent green and red, respectively. Fluorescence was excited with 470 nm of blue light and was detected through an OG 570 high pass colored-glass filter (Zeiss, Thornwood, NY). Each slide was exposed to the excitation light for a period so that the emissions from dead (620 nm) and live (520 nm) cells could be detected for cell counting. For each experiment, internal control data were obtained by counting the number of dead cells that had been incubated with either 5-ALA or Photofrin® but not exposed to laser irradiation.

Lactate Dehydrogenase-Released (LDH) Assay

The CytoTox 96™ assay (Promega Co., Madison, WI) was used to assess the percentage of LDH versus MEC cell lysis after laser irradiation in different concentrations of each photosensitizer. Released LDH in culture supernatants was measured by the conversion of a tetrazolium salt to a red formazan product. The color intensity was proportional to the number of lysed cells. Visible absorbance was read at 490 nm on a THERMO max microplate reader (Molecular Devices, Menlo Park, CA).

Laser-Light Delivery System

Laser irradiation was performed with a Coherent (Palo Alto, CA) Innova 20 argon ion laser

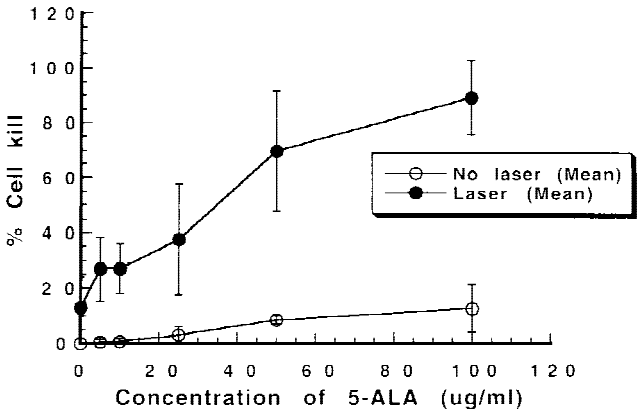


Fig. 3. Photodynamic therapy of microvascular endothelial cells with 5-aminolevulinic acid (5-ALA) in different concentrations show a 50% cell kill at 35 μg/ml.

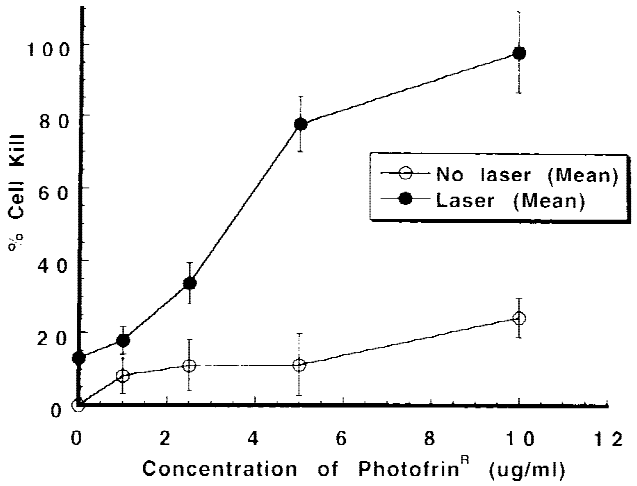


Fig. 4. Photodynamic therapy of microvascular endothelial cells with Photofrin® in different concentrations show a 50% cell kill at 3.5 μg/ml.

pumping a Coherent 599-01 dye laser and was tuned to emit radiation at 630 nm for each experiment. The wavelength was verified with a Jobin Yvon 5/354 UV monochromator (Lonjuneau, France). Irradiation was coupled into a 400-μm fused silica fiberoptic with a Spectra-Physics (Mountain View, CA) Model 316 fiberoptic coupler. The output end of the fiber terminated with a microlens that focused the laser radiation into a circular field of uniform light intensity. Laser irradiation emitted from the fiber was monitored with a Coherent Model 210 power meter before and after laser exposure.

Chicken Animal Model

The highly vascularized chicken comb (Leghorn, Poly Pomona, CA) has been studied extensively. The animals weighed 1,500–2,000 g at the time of PDT. Six chickens were given intravenous injections of either 5-ALA (n = 3) at a drug dosage of 200 mg/kg body weight or Photofrin® (n = 3) at 2.5 mg/kg. Control animals received laser light without the photosensitizer (three areas in one comb), and photosensitizer without laser light (three areas in one comb) was observed.

Three hours after injection of the photosensitizers, the chickens were exposed to the laser (λ = 630 nm). Before illumination, the combs were covered by a metal shield containing a 1.5-cm-diameter circular hole exposing the irradiated area. Total laser energy density was 100 J/cm^2, with a power of 100 mW. Three areas of each comb were treated at power densities of 80, 100, or 120 mW/cm^2 in animals that received either 5-ALA or Photofrin® or the laser only without photosenstizer control groups. The gross changes in chicken combs were recorded immediately, 1 day, 7 days, and 14 days after PDT. All chickens were killed 14 days after PDT, and the combs were processed for histologic evaluation. In addition to examining the wound-healing response of the chicken combs, ultrastructural evidence of blood vessel damage after laser irradiation was sought and documented.

RESULTS

Human Endothelial Cell Culture

The characteristics (von Willebrand factor, CD36) of MEC from both the foreskins and hemangiomas were confirmed to be identical. Subcellular localization of 5-ALA (>35 μg/ml) showed good uptake in the mitochondria (Fig. 1), and Photofrin® (>3.5 μg/ml) showed good uptake throughout the cytoplasm in a diffused pattern (Fig. 2).

Phototoxicity Evaluation by Live/Dead and LDH Assays

Disruption of MT in endothelial cells due to PDT was seen. MEC treated with light (630 nm) plus 5-ALA at a concentration of 35 μg/ml (Fig. 3) and 3.5 μg/ml of Photofrin® (Fig. 4) showed a 50% cell kill. All controls showed a 0% cell kill. The CytoTox 96™ assay for LDH release from MEC after PDT showed a 50% cell kill after laser irradiation for both photosensitizers (Figs. 5, 6).

Animal Histopathology

The average depth of histologic damage was measured in each group of chicken combs (Fig. 7). Chicken combs that received 200 mg/kg of 5-ALA

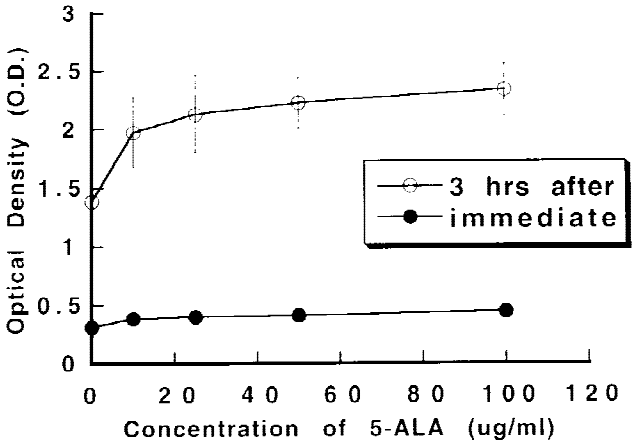


Fig. 5. The CytoTox 96™ assay, in assessing the response of microvascular endothelial cells 3 h later, showed similar results of optical density of lactate dehydrogenase released at LD_{50} concentration of 5-aminolevulinic acid (5-ALA) by laser irradiation.

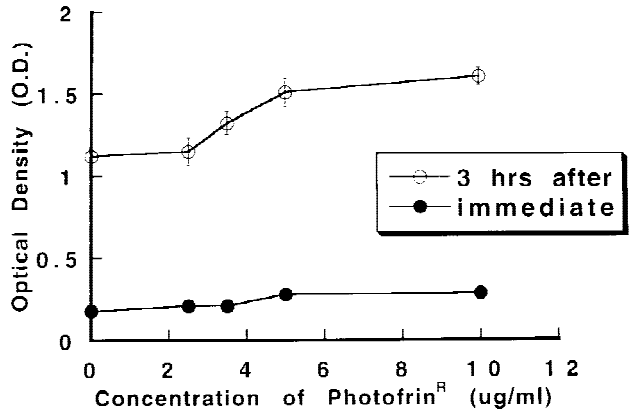


Fig. 6. The CytoTox 96™ assay, in assessing the response of microvascular endothelial cells 3 h later, showed similar results of optical density of lactate dehydrogenase released at LD_{50} concentration of Photofrin® by laser irradiation.

exposed to laser light at 630 nm at a power density of 100 mW/cm^2 had an injury depth of 362.5 ± 27.6 μm at histologic examination. Combs exposed to a power density of 100 or 120 mW/cm^2 showed injury depths of 732.5 ± 29.1 and 792.5 ± 36.0 μm, respectively. Chicken combs that received 2.5 mg/kg of Photofrin® exposed to laser light at 630 nm at a power density of 80 mW/cm^2 had an injry depth of 535.6 ± 22.3 μm at histologic examination. Combs exposed to a power density of 100 or 120 mW/cm^2 showed injury depths of 795.8 ± 32.5 and 805.2 ± 49.1 μm, respectively.

Control chicken combs that received only laser light (80, 100, or 120 mW/cm^2) showed minimal superficial necrosis 14 days later (Fig. 8A). Chicken combs that received 5-ALA without laser

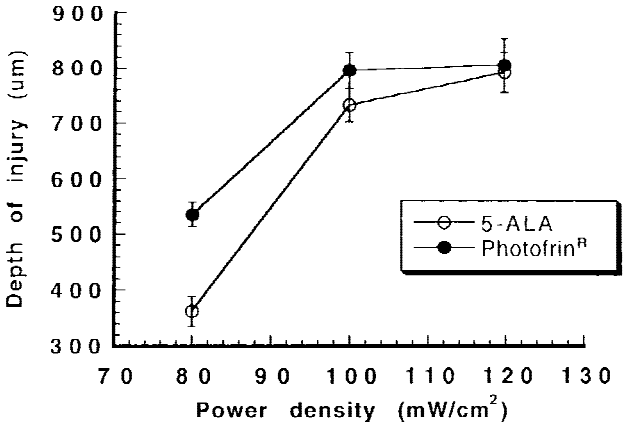
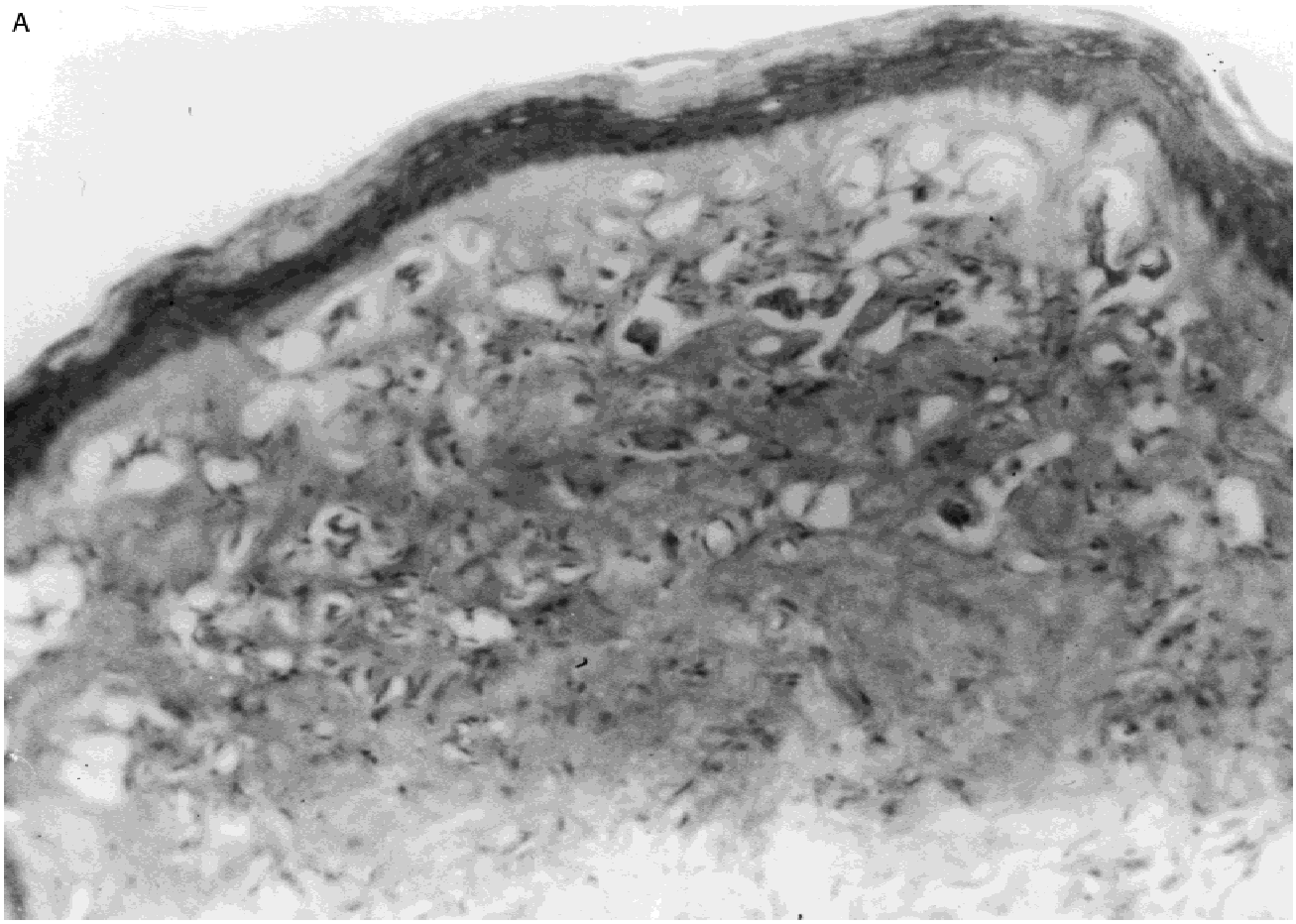


Fig. 7. The depth of injury in a chicken comb after photodynamic therapy with 5-aminolevulinic acid (5-ALA) and Photofrin® by power density.

irradiation showed no gross or histologic damage. Combs that received 5-ALA and were irradiated at a power density of 80 mW/cm^2 showed superficial necrosis on gross inspection 14 days later. Histologic examination of those combs showed only small focal areas of coagulative necrosis adjacent to the surface (Fig. 8B). In the deeper region of the combs, the normal comb architecture was preserved. Combs that received 100 mW/cm^2 showed thicker necrosis at both the gross and histologic levels. In combs that received a power density of 120 mW/cm^2, gross examination showed severe ulcerative necrosis with eschar formation. Histologically, those combs demonstrated almost full-thickness tissue destruction. However, combs that received Photofrin® and 80 mW/cm^2 showed on gross inspection more necrosis in both gross and histologic findings than did the combs that received 5-ALA and were irradiated at a power density of 80 mW/cm^2. In areas that received 100 or 120 mW/cm^2, gross examination showed severe damage with eschar formation. Histologically, those combs demonstrated almost full-thickness tissue destruction.

DISCUSSION

The photosensitizer absorbs photons of the appropriate wavelength and is elevated to an excited state. The excited photosensitizer subsequently reacts with a substrate, such as oxygen, to produce highly reactive singlet molecular oxygen that causes irreversible oxidative damage to biologically important molecules [21,22]. The phototoxic reaction is a local phenomenon that takes place within the same cell on a time scale of



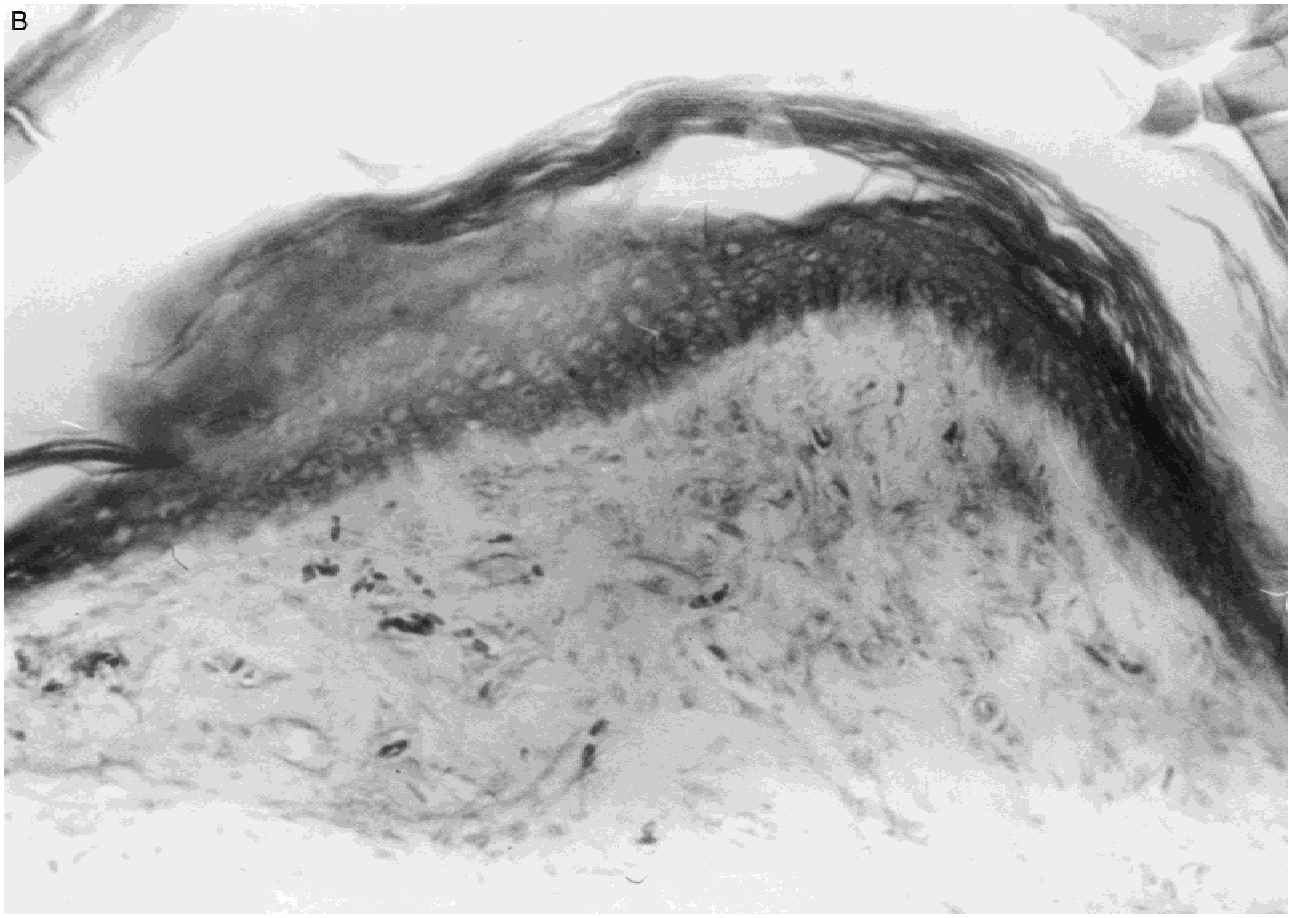


Figure 8

microseconds. Irradiation at the appropriate wavelength absorbed by the photosensitizer provides the energy to drive photodynamic reactions without the generation of heat, provided the incident power density is kept below 100 mW/cm^2 [23].

Efforts to define the mechanisms of PDT action have led to a controversy that attributes cytotoxicity to vascular-mediated events (i.e., indirect cell kill) or to cellular targets (i.e., direct cell kill) of photochemically produced $^1O_2$ or other oxygen radicals [24,25]. It has been hypothesized that if systemic 5-ALA or Photofrin® administration could produce effective vascular photosensitization, the most likely target would be the microvascular endothelial cells [26,27]. Therefore, experiments were performed to ascertain the time course and dose relationship of 5-ALA or Photofrin® in MEC [28]. While these investigations were being conducted, different regiments such as the von Willebrand factor [29] and CD36 [30] were applied to confirm the distinction of cultured MEC from human hemangiomas. The cytoplasmic MT status was monitored by immunofluorescence microscopy by using alpha-tubulin antibody before and after PDT [31]. However, the results after PDT were monitored by calculating the percentage of the MEC cell kill and the optical density of the CytoTox 96™ assay. This approach to defining the mechanisms of PDT action has enabled us to design the animal model for PDT treatment for hypervascular anomalies.

The biochemical results suggest that 5-ALA, located mainly in the inner membrane of mitochondria, is the first committed intermediate in the heme–biosynthesis pathway, which produces an intracellular accumulation of protoporphyrin IX (PpIX). PpIX is an effective photosensitizer, particularly for those reactions involved in oxidative phosphorylation and ATP synthesis associated with skin phototoxicity seen in porphyria patients [32–34]. Photofrin®, located throughout cytoplasm, is highly susceptible to photobleaching during PDT [35,36]. Subcellular localization of 5-ALA and Photofrin® in MEC was detected 3 h after incubation with these photosensitizers. The results in vivo demonstrated marked PDT-induced destruction of the chicken comb. We propose that PDT damage to mitochondrial function and lysosomes could be the major factor responsible for the effectiveness of PDT. However, the higher percentage of Photofrin® versus 5-ALA-derived PpIX located in the cytoplasm of endothelial cells and collagen of chicken combs is consistent with the increased damage by Photofrin®.

Fig. 8. **A:** Control animals that received 80 mW/cm^2 (630 nm) laser light without photosensitizer demonstrate recovery of vasculatures 14 days after laser irradiation. **B:** Focal coagulative necrosis is shown for 5-aminolevulinic acid (200 mg/kg) with 80 mW/cm^2 (630 nm) 14 days after laser light irradiation. Magnification, ×10.

CONCLUSION

Our preliminary study has demonstrated that both 5-ALA and Photofrin® have the ability to destroy MEC in vitro and in vivo. However, continued improvement in treatment results for patients with hypervascular cutaneous anomalies will depend on the ability to cause selective destruction of only the targeted blood vessels buried deep within the skin without the production of heat by nonthermal mechanisms. In addition, the greater tissue penetration of the longer wavelengths used in PDT should make it ideal for the treatment of deeper, larger hemangiomas, thus substantially expanding the population of patients expected to benefit from laser treatment.

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